SOME PROPERTIES OF SPERM EXTRACTS AND THEIR RELATIONSHIP TO THE FERTILIZATION REACTION IN ARBACIA PUNCTULATA[1]

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Since the early work of Piéri (1899), numerous attempts have been made to isolate substances from sperm which could be shown to function in normal fertilization. Extracts and filtrates of sperm, prepared by a wide variety of methods, have consistently proved inactive as parthenogenetic agents. The literature of this subject has been reviewed by Sampson (1926), who points out that any positive results reported may be ruled out on the basis of technical or interpretive errors. Largely because of these discouraging results, the search for the hypothetical "fertilizing substance" of sperm has been abandoned during the past decade.

The purpose of this investigation was to study certain properties of sperm extracts obtained by heating and filtering sperm suspensions of *Arbacia*. The experiments deal with (1) the agglutinating action of sperm extracts on *Arbacia* eggs, (2) the relation of such extracts to egg-water ("fertilizin" of Lillie, 1913, etc.), (3) the effects of sperm extracts on the fertilizing power of sperm, the fertilizability of eggs, and the development of fertilized eggs, and (4) the action of sperm extracts as parthenogenetic agents.

Materials and Methods

The experiments were carried out during the summers of 1934, 1937, and 1938 on the gametes of the sea-urchin, *Arbacia punctulata*. Eggs and sperm were obtained by the method described by Just (1928). After cutting out the lantern and peristome with scissors, the coelomic fluid was washed away with sea water and the urchin placed aboral side down over a Stender dish and allowed to shed. Mixed "dry" sperm from several males was kept in a test tube until required and mixed shed eggs were washed and placed in a small volume of sea water. Where large amounts of material were needed or when the

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animals shed poorly, the ripe gonads were removed with forceps, washed, and strained through cheesecloth. All instruments and glassware were rinsed thoroughly in tap and sea water before use to prevent chance contamination of eggs with sperm.

In preparing jellyless eggs, shed ("normal") eggs were treated with a mixture of 1.4 cc. N/10 HCl in 50 cc. sea water, washed, and examined in Chinese ink to insure the absence of jelly (Lillie, 1915*a*). This procedure removes the jelly from 90–100 per cent of the eggs without harming them.

Sperm extracts were prepared as follows: Sperm suspensions, made up in 10-cc. portions by adding a measured volume of dry sperm to sea water, were heated one minute over a small Bunsen flame. The albuminous sperm coagulum was removed by filtration through No. 5 Whatman filter paper. The filtrate, or sperm extract, was considered to be equal in concentration to that of the sperm suspension from which it was obtained. Thus a 10 per cent sperm extract was prepared by heating and filtering 1 cc. sperm in 9 cc. sea water.

In preparing and determining the fertilizin concentration of egg-water, Lillie's method (1914) was followed with slight modifications. Fresh unfertilized *Arbacia* eggs, which had stood at least fifteen minutes in a small volume of sea water, were lightly centrifuged and the supernatant fluid (egg-water) removed with a pipette. The presence of fertilizin was detected by adding a drop of egg-water to a drop of fresh 1 per cent sperm suspension and observing reversible agglutination of the sperm under the microscope. By making successive half-dilutions of the egg-water with sea water, a dilution is reached at which the sperm agglutinate for three to six seconds but above which no agglutination occurs. This dilution, by definition, contains one fertilizin unit and the fertilizin concentration of the original egg-water is thereby determined. For example, if an egg-water agglutinates sperm at 1/1600 dilution but not at 1/3200, the undiluted egg-water contains 1600 fertilizin units.

The Egg-Agglutination Reaction

When a few drops of *Arbacia* eggs are added to a small quantity of sperm extract in a tube, a striking reaction occurs. As each drop falls it sets into a dense red mass which remains suspended from the surface of the solution by a thin ribbon of coagulated eggs (Fig. 1, *A*). When the tube is shaken the ribbons coalesce to form a loose clot of eggs which usually floats at the surface of the extract (Fig. 1, *B*). A similar quantity of eggs, shaken in heated water as a control, forms

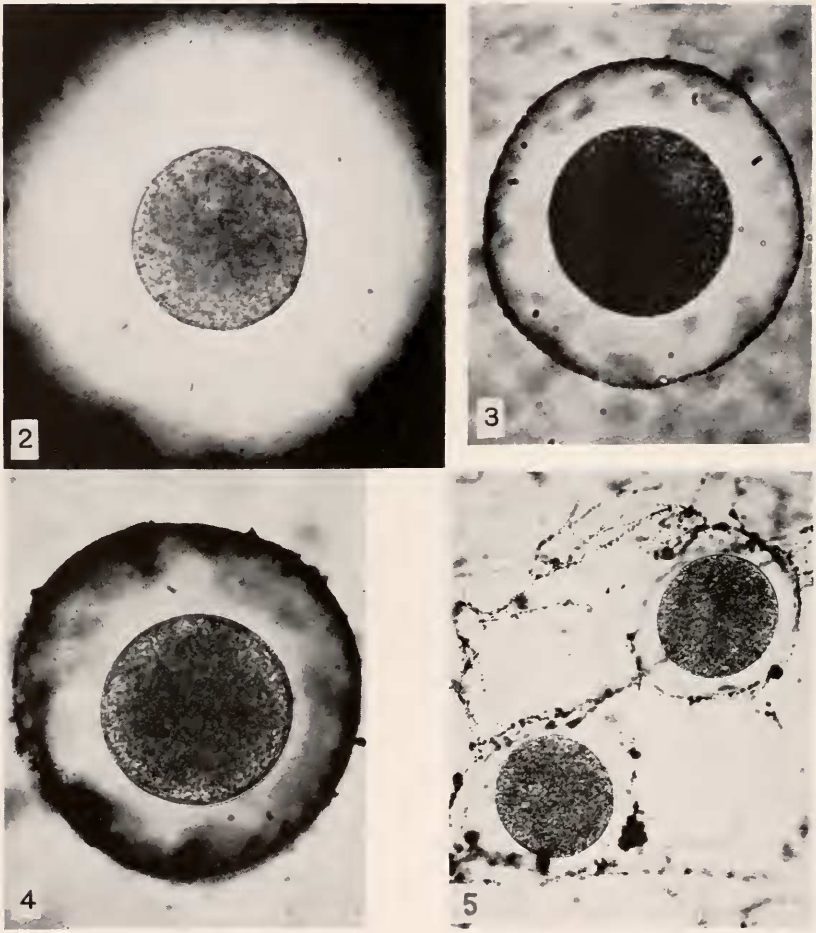

PLATE I

Microscopic appearance of the egg-agglutination reaction. All eggs photographed in Chinese ink.

FIG. 2. Normal *Arbacia* egg in heated sea water surrounded by a wide zone of clear, colorless jelly. × 430.

FIG. 3. A similar egg after five minutes exposure to a 5 per cent sperm extract. Note that a distinct agglutination membrane has appeared at the periphery of the jelly. × 430.

FIG. 4. A similar egg after thirty minutes exposure to a 5 per cent sperm extract. The agglutination membrane has become denser but the jelly immediately in contact with the cortex remains unaffected. × 430.

FIG. 5. Strands of agglutinated jelly connecting eggs in 5 per cent sperm extract. × 215.

a homogeneous suspension which slowly settles (Fig. 1, *C*). Subsequent agitation of each tube causes an increase in the density of the egg-masses in the extract, while the eggs in sea water redistribute themselves through the solution and again settle to the bottom of the tube. We will call this phenomenon the "egg-agglutination reaction," and the inciting substance present in sperm extracts, the egg-agglutinating substance. These terms are used in a purely descriptive sense without implying an analogy to immunological agglutinations.

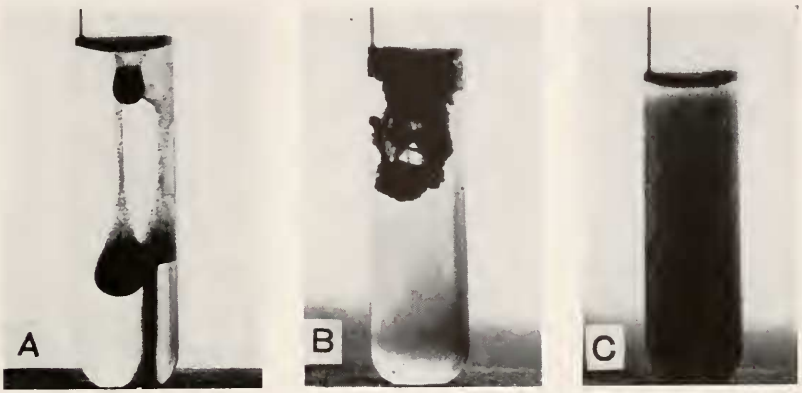

FIG. 1. Gross appearance of the egg-agglutination reaction. All photographs natural size.

(A) Five drops *Arbacia* eggs in 3 cc. of 5 per cent sperm extract. Note the drops of agglutinated eggs suspended from the surface of the solution.

(B) Ten drops of *Arbacia* eggs in 3 cc. of 5 per cent sperm extract. The tube was shaken once. The eggs have formed a large dense mass which floats at the surface.

(C) Ten drops of *Arbacia* eggs in 3 cc. heated sea water. The tube was shaken once after adding the eggs. Observe that the ova are homogeneously suspended and have begun to settle as shown by the clear area immediately below the surface.

The details of the egg agglutination reaction may be observed microscopically by making an egg suspension in Chinese ink which reveals the colorless jelly layer, or chorion, surrounding each ovum. By means of a capillary pipette, sperm extract is introduced into a drop of this mixture mounted on a slide beneath a raised coverslip and observed under high power. As the extract comes in contact with an egg, a faint black line of adhering ink particles immediately appears at the periphery of the jelly. This line rapidly increases in thickness until the chorion is encircled by a dense membranous structure which will be termed the "agglutination membrane."

The microscopic appearance of eggs treated with sperm extract is shown in Plate I, Figs. 2–5. A normal egg is surrounded by a wide zone of clear colorless jelly with poorly-defined margins (Fig. 2). On treating such an egg with sperm extract, a distinct agglutination membrane forms at the periphery of the jelly (Fig. 3), which gradually increases in density (Fig. 4). Particles of jelly freely suspended in the solution also agglutinate to form a dense interlacing network connecting adjacent eggs (Fig. 5).

With these changes the jelly becomes very adhesive, causing the ova to stick firmly together in clusters when the coverslip is moved about. Woodward (1915) states that the jelly of *Arbacia* eggs placed in "boiled sperm suspension" swells and becomes sticky so that the eggs adhere to each other and to the container. This observation was repeatedly confirmed during the present study with the exception that swelling of the jelly was never noted, either in boiled sperm suspensions or in sperm extracts. In some instances the chorion retains its original width but it generally shrinks markedly, as shown by a decrease in the space between agglutination membrane and cortex after prolonged exposure to the solution.

The egg-agglutinating substance affects only that portion of the jelly with which it comes in contact and incomplete agglutination membranes involving one-half or less of the circumference of the chorion appear on those eggs which have been only partially exposed to the extract.

In general, the density and rate of formation of the agglutination membranes are directly related to the concentration of extract. A 4 per cent sperm extract clots the eggs immediately into a solid mass surrounded by a thick unbroken line of agglutinated jelly; as the extract decreases in concentration, fewer eggs are involved, the reaction is slower, and the membranes are thinner until below about 0.5 per cent both gross and microscopic reactions become negative.

The egg-agglutination membranes grow tougher and more resistant to tension with time. After a few minutes exposure to a 2 per cent extract, the coverslip may be gently agitated without altering the circular shape of the membranes and the eggs move about freely in the unaffected central jelly. When greater tension is applied by rapidly agitating the coverslip, the membrane ruptures and the ovum may then be torn away leaving the membrane firmly anchored to the slide with its frayed edges waving about in the solution.

The egg-agglutination reaction is irreversible. Eggs which have once agglutinated remain firmly massed together in spite of transfer to sea water and eventually cytolyse *in situ*. Vigorous shaking increases

the size and density of the egg-clusters and leads to rapid destruction of the ova.

The jelly of *Arbacia* eggs binds the egg-agglutinating substance and removes it from the extract. This is illustrated by a typical experiment: 1 cc. shed eggs was added to 3 cc. of 20 per cent sperm extract and the agglutinated egg-mass was filtered off. The filtrate weakly agglutinated 1 cc. eggs, after which the solution was again filtered but this filtrate failed to agglutinate .5 cc. eggs. Three cc. of the same extract diluted with 2.5 cc. sea water as control strongly agglutinated eggs. The egg-agglutinating substance present in the 20 per cent extract has, therefore, been fixed by the jelly of 2 cc. eggs.

Sperm extracts not only act on the jelly but also on the cortex of *Arbacia* eggs. To study this, jellyless eggs were divided into two lots, one of which was fertilized with fresh sperm. Samples from each lot were fixed in Bouin's solution (without acetic acid) or 2 per cent osmic acid and then washed thoroughly in sea water. Jellyless eggs treated with sperm extract form clusters which are too minute to be reliably detected grossly so a microscopic method was used: a drop of jellyless eggs was mixed with a few drops of extract on a slide and a glass needle was moved through the mixture. The appearance of distinct egg-clusters represented a positive agglutination test.

Numerous experiments demonstrated that the cortex undergoes certain changes in sperm extracts causing the eggs to fuse so firmly together that bits of cytoplasm may be ripped out of the ovum by agitating the egg-clusters. In addition the fertilization membranes of fertilized jellyless eggs adhere to each other and may be pulled completely away from the zygote. These changes take place equally in unfertilized and fertilized jellyless eggs whether living or dead. The structure of the cortex, however, does not appear to be visibly altered by exposure to sperm extracts. Controls of the same eggs in heated sea water remained singly spaced in all cases, except for occasional small clusters caused by the use of acid in removing the jelly (Lillie, 1915*a*).

Identical experiments on normal eggs showed that the jelly of fertilized and unfertilized eggs, both living and dead, agglutinates in sperm extracts. Immature ovocytes, with or without jelly, agglutinate as readily as mature ova.

To sum up: (1) The egg-agglutination reaction is not dependent on living protoplasm, since dead ova agglutinate as readily as living ones. (2) Immature, unfertilized, and fertilized eggs agglutinate equally, indicating that the developmental state of the ovum plays no part in the reaction. (3) Sperm extracts agglutinate jellyless eggs by directly affecting the cortex which becomes markedly adhesive.

One of the first questions to arise was whether the egg-agglutinating substance could be extracted from tissues or cells of *Arbacia* other than sperm. Extracts of *Arbacia* shells, lanterns, peristomes, ovaries, eggs, and coelomic fluid, prepared by heating 4 cc. of each tissue one minute in 10 cc. of sea water, were found to be without effect on ova. Extracts of immature testes or ripe testes washed free of sperm were likewise inert. The egg-agglutinating substance is thus highly tissue-specific and can be extracted solely from sperm. Moreover, sperm extracts act specifically on *Arbacia* eggs and will not agglutinate blood corpuscles, spermatozoa or any other cells of the male or female sea-urchin.

The egg-agglutination reaction is not confined to the gametes of *Arbacia* and a certain degree of cross-agglutination exists between eggs and sperm extracts of related and distant species. Preliminary experiments on the gametes of *Nereis* and *Echinarachnius* gave the following results: (1) *Echinarachnius* sperm extracts agglutinate ova of *Echinarachnius* and *Arbacia*. (2) *Nereis* sperm extracts agglutinate *Arbacia* eggs but do not agglutinate fertilized or unfertilized *Nereis* eggs. In *Nereis*, jelly extrusion occurs after fertilization, hence both fertilized and unfertilized ova were tested for agglutination. Just (1922) found that *Nereis* sperm boiled in sea water causes a small percentage of jelly formation, maturation, and differentiation without cleavage of unfertilized *Nereis* eggs. (3) Sperm extracts of *Arbacia* agglutinate *Echinarachnius* eggs. Such extracts will not agglutinate *Nereis* eggs but cause jelly extrusion and maturation (without cleavage) of unfertilized *Nereis* eggs.

The chemical specificity of the egg-agglutination reaction is less distinct than the biological. *Arbacia* eggs are agglutinated by certain salts, foreign sera (Robertson, 1912), acids and alkalis.

Physical and Chemical Properties of the Egg-Agglutinating Substance

These experiments were devised to study certain general physical and chemical properties of the egg-agglutinating substance and do not represent exact quantitative studies. Each experiment was repeated several times with different materials and the results were consistent in each case. The egg-agglutination tests were standardized by adding three drops of fresh eggs to 2 cc. of sperm extract in a test tube and shaking the tube lightly. In certain experiments the agglutinating strength of the extracts was rated as follows:

0 = No agglutination. All eggs homogeneously suspended.
1 = Few thin shreds of agglutinated eggs with the majority freely suspended.
2 = One or more loose clusters of agglutinated eggs with some ova freely suspended.
3 = Single dense clot of agglutinated eggs with no eggs free in the solution (maximum).

Appearance, Specific Gravity, and pH of Sperm Extracts

Extracts of clean shed sperm are colorless while extracts of testis sperm have a light purple tint ("purple X" of Glaser, 1914). Dilute sperm extracts are clear; those of high concentration (40 per cent) are faintly opalescent. The specific gravity of a 50 per cent extract is 1022, which about equals that of sea water (av. sp. gr. = 1028). The pH of a 60 per cent sperm extract in sea water, measured with the Coleman glass electrode, is 7.74; that of a 1 per cent extract is 8.02. The slight relative acidity of the 60 per cent extract probably results from CO_2 liberated by the thick sperm suspension prior to extraction. These values approximate the pH of sea water at Woods Hole (av. pH = 8.00).

Relation of Temperature to the Extraction and Stability of the Egg-agglutinating Substance

To determine whether the egg-agglutinating substance can be extracted from sperm at low temperatures, 10 cc. sea water was heated in a waterbath to a given temperature, a measured amount of mixed shed sperm was stirred into the sea water and kept for one minute at the desired temperature, after which the solution was filtered. Under these conditions it was found that sea water below about 57° C. will not extract the egg-agglutinating substance. This can be extracted in small amounts from a 20 per cent sperm suspension at 57° and from a 1 per cent suspension at 68° C. Higher temperatures extract greater quantities of the substance from sperm. There appears to be an inverse relation between sperm concentration and the critical temperature of extraction.

The rate of loss of the egg-agglutinating substance on standing is a function of the temperature of the extract: 50 cc. of a 10 per cent sperm extract was divided into two equal parts, one of which was aged at room temperature (26° C.) and the other in an icebox (7° C). The results of tests on each solution at various times are given in Table I.

The agglutinating strength of the extract kept at 7° C. remained constant for twenty-five hours and then gradually diminished and dis-

appeared after forty-five hours. The same extract at 26° C. lost its activity in less than thirteen hours. In a similar experiment, a 1 per cent extract lost its agglutinating power in less than twelve hours at 26° C. and in twenty hours at 7° C. showing that the egg-agglutinating substance disappears more rapidly from dilute extracts. We may conclude that this substance is preserved best at low temperatures.

The egg-agglutinating substance is highly heat-resistant and only slowly destroyed by boiling: 25 cc. of a 20 per cent sperm extract was boiled for several hours in a beaker, the volume being kept constant by addition of distilled water. The agglutinating strength of the extract remained high (3) for four hours, then gradually decreased, and dropped to zero after five and one-half hours of boiling.

TABLE I

Egg-agglutinating strength of a 10 per cent sperm extract aged at 7° C. and 26° C. (3 = maximum)

Age of Extract	Temperature 7° C.	26° C.
hours		
0	3	3
13	3	0
25	3	—
38	2	—
41	1	—
45	0	—

Dialysis and Filtration

Thirty cc. of a 30 per cent sperm extract was divided into three equal parts (*A, B,* and *C*), each of which was placed in a Thomas Diffusion Shell (No. 4471). Extract *A* was dialysed against running sea water in a beaker, *B* was suspended in a cylinder containing 10 cc. sea water, and *C* was kept as a control. After twelve hours each extract was tested for its activity. If dialysis had occurred, one would expect a decrease in the concentration of the egg-agglutinating substance in extracts *A* and *B* and the appearance of this substance in the dialysate of extract *B*. By dilution of each solution with sea water, however, it was found that the concentration of the substance in *A* and *B* exactly equalled that of the control, *C*. The dialysate of *B* was totally inert. Numerous similar experiments proved conclusively that the egg-agglutinating substance is non-dialysable. Also it will not pass through a Berkfeld filter regardless of the concentration of sperm extract. These facts suggest that it is a colloidal substance of large molecular size.

Source of the Egg-agglutinating Substance

The sperm heads of *Arbacia* are composed chiefly of nucleic acids and "Arbacin," a substance having properties in common with both histones and protamines (Mathews, 1897). In view of the relatively minute amounts of lipids which are confined largely to the sperm tails, it seemed likely that the egg-agglutinating substance might be a protein or protein derivative. Sperm extracts were found to be negative to the following tests for proteins: Millon's, Adamkiewicz, biuret, xanthoproteic, and ninhydrin. No precipitate forms on adding saturated $(NH_4)_2SO_4$ or 70 per cent alcohol and no reduction occurs with Benedict's solution or bismuth subnitrate. Hence if proteins or carbohydrates are present in sperm extracts, their concentration is too minute to be detected by ordinary chemical tests.

The lipids of sperm may be readily separated from the proteins by alcohol-ether extraction. The procedure used was adapted after Bloor's method for extraction of total lipids from blood serum (Peters and Van Slyke, 1932, p. 495). Ten cc. of mixed shed sperm was shaken in 150 cc. alcohol-ether mixture (three parts 95 per cent alcohol plus one part ethyl ether) and the mixture boiled by immersion in a waterbath. The solution was made up to 200 cc. with alcohol-ether, cooled, and filtered through fat-free filter paper. The rubbery pink protein residue was washed thoroughly in sea water, heated one minute in 10 cc. sea water and filtered. The filtrate strongly agglutinated eggs.

The honey-colored lipid extract was evaporated to dryness on a waterbath, the residue was dissolved in 100 cc. petroleum ether, filtered, and re-evaporated to dryness. This procedure yielded a small quantity of yellow waxy material which was shaken in 10 cc. sea water, heated one minute, and filtered. The filtrate had no effect on eggs.

To determine whether heat was the factor responsible for liberating the egg-agglutinating substance in the above procedure, shed sperm was shaken in cold alcohol-ether solution, after which the protein precipitate was washed in sea water, shaken vigorously in 10 cc. sea water, and filtered. The filtrate markedly agglutinated eggs proving that heat is not essential for extraction.

To sum up, the egg-agglutinating substance is derived from the protein residue (head) of the spermatozoön and is not found in the lipid fraction.

Chemical Composition of the Extraction Medium

Sperm extracts made in tap or distilled water will not agglutinate eggs and if the residue from a sperm suspension, which has been heated

one minute in sea water, is reheated in distilled water, the extract is likewise inert. By substituting sea water for distilled water, in the above procedures, the egg-agglutinating substance is readily obtained. These facts suggested that certain salts or ions, present in sea water, are essential for the extraction of the substance. A thorough study of this problem requires chemical analyses of various extraction media and is beyond the scope of this investigation which is concerned with reactions occurring in sea water. The experiments cited below, therefore, represent preliminary studies:

Ten per cent sperm suspensions were made up in each of the following solutions and extracted by heating one minute and filtering:

1. Van't Hoff artificial sea water solutions each of which lacked a single salt present in normal sea water: $CaCl_2$, $MgSO_4$, $MgCl_2$, KCl, and NaCl.

2. Pure solutions of each of the above salts in distilled water.

3. Pure solutions of Mg, Ca, K, and Na acetate in distilled water. The reagents were all made up isosmotic with sea water and the pH varied from about 7.2–9.0. (The viscosity, ionic concentration, and molarity could not be controlled.) None of the solutions visibly injured ova or sperm, or agglutinated eggs.

It was found that an active sperm extract agglutinated eggs washed in any of the above media to the same degree as eggs washed in sea water. Shed eggs in sea water were therefore used in the egg-agglutination tests.

From the results, listed in Table II, the following conclusions may be drawn:

(1) Sea water lacking magnesium ($MgCl_2$, $MgSO_4$ or both) will not extract the egg-agglutinating substance from sperm whereas pure solutions of $MgCl_2$ or $MgSO_4$ will. Magnesium is thus essential to the extraction process.

(2) Sea water lacking calcium gave variable results; out of 6 tests, 3 were weakly positive and 3 negative. A pure solution of $CaCl_2$ readily extracts the egg-agglutinating substance. Calcium, therefore, appears to be necessary in sea water for the extraction of this substance.

(3) Sperm extracts made in sea water which lacks either NaCl or KCl agglutinate eggs. Extracts made in pure NaCl or KCl solutions have no effect on eggs. These salts evidently play no part in the extraction of the egg-agglutinating substance in sea water.

(4) If acetate ions are substituted for chloride ions in pure solutions of the salts of sea water, it is found that Mg and Ca acetate will extract the egg-agglutinating substance from sperm but Na and K

acetate will not. The egg-agglutination reaction is therefore not dependent upon the presence of any particular anions.

To sum up, the bivalent cations, magnesium and calcium, are the only ions of *sea water* which are necessary for the extraction of the egg-agglutinating substance from sperm. Both of these ions must be present in sea water but each will act alone when present in sufficient concentration in distilled water.

Extraction of Sperm in Acid and Alkaline Sea Water

In these experiments .10 N HCl and .10 N NaOH were diluted with varying quantities of sea water and the solutions used in preparing 5 per cent sperm extracts.

TABLE II

The agglutination of eggs by 10 per cent sperm extracts prepared in various isosmotic salt solutions. Three drops of eggs added to 2 cc. of each extract. + = positive tests. 0 = negative tests. All solutions prepared by Marine Biological Laboratory Chemical Room.

Extraction Solution	Egg-agglutination
* Artificial Sea Water (Van't Hoff)	+
* Ca-free Sea Water	+ or 0
* Mg-free Sea Water	0
* $MgSO_4$-free Sea Water	0
$McCl_2$-free Sea Water	0
KCl-free Sea Water	+
* NaCl-free Sea Water	+
.34 M $CaCl_2$	+
* .37 M $MgCl_2$	+
* $MgSO_4 \cdot 7H_2O$	+
.53 M KCl	0
.52 M NaCl	0
Ca Acetate	+
Mg Acetate	+
K Acetate	0
Na Acetate	0

* Indicates extracts on which fertilizin-inactivation tests were run with results parallel to those for egg-agglutination tests.

Lillie (1915*a*) states that acid sea water heavily agglutinates and later cytolyses *Arbacia* eggs. The present study shows that when sperm is extracted in acid sea water of above .003 N HCl content, the agglutinating effect of the acid hides the action of the egg-agglutinating substance. These substances, however, have different actions since HCl agglutinates and later cytolyses eggs whereas the egg-agglutinating substance agglutinates but does not kill the ova. Sperm extracts in sea water of or below .003 N HCl content agglutinate eggs. Sea water of

this degree of acidity has no effect, which proves that the agglutination is here caused by the egg-agglutinating substance alone.

Haas (1916), Irving (1926), and Kapp (1928) have shown that the addition of .10 N NaOH to sea water precipitates most of the magnesium and some calcium as hydroxides. In the present study it was found that alkaline sea water containing more than .01 N NaOH loosely flocculates *Arbacia* eggs, probably due to precipitation of calcium and magnesium hydroxide in the egg jelly. This precipitate is removed when alkaline sea water is heated and filtered (as in preparing sperm extracts) and eggs added to the filtrate remain singly spaced. It is thus possible to determine the highest concentration of alkali in sea water which will permit the extraction of the egg-agglutinating substance from sperm. This was found to be about .012 N NaOH sea water; above this degree of alkalinity no agglutination takes place; below it the extracts agglutinate eggs. The previous section has shown that sea water lacking Mg or Ca will not extract the egg-agglutinating substance from sperm. According to Irving (1926), .017 molar NaOH precipitates 19 per cent of the total Ca and 16 per cent of the total Mg from sea water. A possible explanation for the action of alkaline sea water in extracting sperm is that sufficient Mg and Ca are removed from sea water by concentrations of alkali above .012 N NaOH to render it inert as an extracting medium. Below this degree of alkalinity, however, enough ionized Mg and Ca are present to allow extraction of the egg-agglutinating substance.

The pH of the solutions varied from 3.2 (.003 N HCl in sea water) to 9.5 (.012 N NaOH in sea water). Since sperm extracts made in either of these solutions agglutinate eggs, the egg-agglutination reaction takes place over a wide range of hydrogen-ion concentrations.

The Inactivation of Fertilizin by Sperm Extracts

As is well known from the classical studies of F. R. Lillie (1913–1919), mature unfertilized eggs of *Arbacia* secrete a substance into sea water which reversibly agglutinates sperm of the same species. Lillie named this substance "fertilizin" and concluded that it functions in fertilization as an essential chemical link in the union of egg and spermatozoön. For further details concerning the fertilizin theory of fertilization see the recent review of Just (1930).

Since fertilizin is present in high concentration in the jelly of *Arbacia* eggs, it seemed probable that sperm extracts might react in some way with this substance, which proved to be correct. When sperm extracts are mixed with egg-water, the latter loses its capacity to agglutinate sperm; some substance in the extract has neutralized the

fertilizin. For description purposes we will call this " fertilizin-inactivation " and the hypothetical substance responsible for it the " fertilizin-inactivator."

To study the relation of fertilizin-inactivation to the concentration of sperm extract, 2 cc. of successive half-dilutions of an 8 per cent extract was placed in a series of test tubes and 2 cc. egg-water of known fertilizin content was added to each tube. This procedure was repeated with egg-waters of descending fertilizin content, the concentration of sperm extracts remaining constant. The highest dilution of egg-water was mixed with an equal quantity of heated sea water as a control. A drop of each mixture was tested for fertilizin by its ability to agglutinate a drop of fresh 1 per cent sperm suspension; absence of sperm agglutination constituted a positive fertilizin inactivation test; agglutination of sperm, indicating the presence of free fertilizin in the mixture, was considered a negative fertilizin-inactivation test.

In Table III, which summarizes the results of three experiments, a change from + to 0 in each horizontal row represents the end-point at which the extract in the corresponding vertical column becomes too dilute to inactivate all of the fertilizin present. For example, 400 fertilizin units are inactivated by a 4 per cent but not by a 2 per cent sperm extract. In each experiment at least three determinations were made at each of these end-points with different sperm suspensions as indicator in order to reduce error caused by variation in materials. Note (Table III) that an 8 per cent extract inactivates 800 fertilizin units whereas a .06 per cent extract only inactivates 10; a .1 per cent extract inactivates 20 but not 40 fertilizin units. The controls agglutinated sperm in all instances. Sperm extracts thus inactivate fertilizin in roughly quantitative proportions, the capacity for inactivation varying directly with the concentration of extract.

Certain incidental phenomena were noted during the course of this experiment. When a drop of concentrated sperm extract plus egg-water is added to a drop of spermatozoa, the latter form a dense, highly active ring around the introduced drop but sperm agglutination does not occur. Sperm extracts therefore react only with fertilizin without altering the " sperm-activating and aggregating " substances of egg-water, which confirms Lillie's statement (1914) that these substances are distinct from fertilizin. Furthermore, it was noted that on shaking concentrated sperm extract plus egg-water mixtures, a light purple, flocculent precipitate appears and the egg-water, which previously was purple, immediately becomes colorless. The precipitate floats at the surface of the solution and is probably formed by agglutination of sub-microscopic jelly particles present in the egg-water.

It may be pointed out here that fertilizin does not appear to be essential to the egg-agglutination reaction, since fertilized jellyless eggs washed free of fertilizin agglutinate as readily as unfertilized jellyless eggs which secrete large quantities of this substance.

Table III

Combined results of three experiments on the inactivation of fertilizin by sperm extracts. Upper horizontal column gives concentration of sperm extracts; vertical column gives concentration of egg-water in units of fertilizin. For each test, 2 cc. egg-water was added to 2 cc. extract and the mixture tested for its ability to agglutinate a 1 per cent sperm suspension. + = positive tests (no sperm agglutination); 0 = negative test (sperm agglutination).

Concentration of Sperm Extract:	8%	4%	2%	1%	0.5%	0.2%	0.1%	0.06%	0.03%
Fertilizin Units	Fertilizin-Inactivation Tests								
800	+	+	0	0	0				
	+	+	0	0	0				
	+	+	0	0	0				
400	+	+	0	0	0	0			
		+	0	0	0				
200	+	+	+	0	0	0			
		+	+	+	0	0			
			+	+	0	0			
100		+	+	+	0	0			
		+	+	+	0	0			
80			+	+	+	0	0		
			+	+	+	0	0		
			+	+	0	0	0		
40			+	+	+	0	0		
			+	+	+	+	0		
				+	+	0	0		
20					+	+	+	0	
				+	+	+	+	0	
				+	+	0	0	0	
10					+	+	+	+	+
					+	+	+	+	0

Properties of the Fertilizin Inactivator

We have described two effects of sperm extracts, one on eggs and the other on fertilizin. To determine whether these effects are related

to each other, simultaneous tests for the egg-agglutinating substance and fertilizin-inactivator were run on sperm extracts. The fertilizin-inactivation tests represent a part of the previously described experiments dealing with the properties of the egg-agglutinating substance and the results of the former will, therefore, be briefly summarized without repeating experimental details. The fertilizin-inactivation tests were standardized by adding .3 cc. sperm extract to .3 cc. egg-water of known fertilizin content and testing the mixture for agglutination on a fresh 1 per cent sperm suspension. The inactivating strength of a sperm extract was considered equal to the maximum number of fertilizin units which it neutralized.

The fertilizin-inactivator is markedly tissue-specific and cannot be extracted from any tissue or cells of *Arbacia* except sperm. It has long been known that blood and tissue extracts of *Arbacia* will not combine with fertilizin but that extracts of washed unfertilized eggs contain a substance, "anti-fertilizin," which neutralizes minute amounts of fertilizin (Lillie, 1914). Because anti-fertilizin combines only with fertilizin without affecting the sperm-aggregating and activating factors of egg-water, it acts like the fertilizin-inactivator, but the latter is much more potent in neutralizing fertilizin.

Table IV

Fertilizin-inactivating strength of a 10 per cent sperm extract aged at 7° C. and 26° C. Inactivating strength given as maximum number of fertilizin units inactivated by extract.

Age of Extract	Temperature 7° C.	26° C.
hours		
0	400	400
13	400	50
25	200	—
38	100	—
41	50	—
45	25	—

Low temperatures preserve the fertilizin-inactivator as shown in Table IV, which gives the data from a single experiment. After thirteen hours at 26° C. the inactivating strength of a 10 per cent extract dropped from 400 to 50; after twenty-five hours at 7° C. the inactivating strength fell from 400 to 200 and then gradually decreased to 25 in forty-five hours.

The fertilizin-inactivator is quite thermostable and is gradually destroyed by boiling. The inactivating strength of a 20 per cent sperm

extract fell from 400 to 50 after two and one-half hours at 100° C. and remained at 50 after five and one-half hours of boiling.

It can be obtained from the protein residue but not from the lipid fraction when sperm is extracted with hot alcohol-ether and it is extractable from sperm shaken in cold alcohol-ether. It will not dialyse or pass through a Berkfeld filter and is not present in sperm extracts made in tap or distilled water. In the previously described experiments with artificial sea water solutions, fertilizin-inactivation tests were run on a few of the extracts (marked with a (*) in Table II) and the results paralleled the egg-agglutination tests in each case.

Because the egg-aggglutinating substance and fertilizin-inactivator in general showed parallel behavior in all experiments they will be referred to from now on collectively as the "active principles" of sperm extracts.

Secretion of Active Principles and their Relation to the Fertilizing Power of Sperm

Filtrates of fresh or old sperm suspensions which have been passed through Whatman filter paper and the supernatant fluid of centrifuged sperm suspensions will not react with fertilizin or eggs. The active principles are found only in sperm *extracts* and are not secreted by sperm into water.

It was found that extracts of sperm which have been aged until dead are inert. The fact that the active principles can only be extracted from fresh sperm suggested that they might be related to the fertilizing power of sperm. Experiments were therefore undertaken to determine whether the loss of fertilizing power of aging sperm suspensions bears any relation to the loss of active principles from extracts of these suspensions. Sperm suspensions of various concentrations (8 per cent, 4 per cent, 2 per cent, and 1 per cent) were prepared by adding measured amounts of mixed shed sperm to 80 cc. sea water in a series of Erlenmeyer flasks. The sea water was sterilized in an autoclave to lessen bacterial growth which shortens the life of sperm (Cohn, 1918). The flasks were closed with sterile corks and aged at room temperature (26°–28° C.) for three days. At intervals 4 cc. of each suspension was heated thirty seconds, filtered, and the extract divided into two equal parts which were tested for the active principles.

In the fertilizin-inactivation tests, in order to insure complete inactivation of fertilizin by each extract at the start of the experiment, descending concentrations of fertilizin were added; 200 fertilizin units were added to the 8 per cent, 100 to the 4 per cent, 50 to the 2 per cent and 25 to the 1 per cent sperm extract.

Simultaneously with these tests, the fertilizing power of the sperm was measured by adding one drop from each sperm suspension to three drops of fresh eggs in 10 cc. sea water and counting the percentage of fertilized eggs in each dish two hours later.

Table V

The relation between loss of fertilizing power of aging sperm suspensions and loss of active principles from extracts of these suspensions. Horizontal rows give simultaneous tests made at intervals recorded at left. *A* = egg-agglutination tests. *B* = fertilizin inactivation tests; the figures in brackets are the number of fertilizin units added to each extract. *C* = fertilizing power of sperm suspensions expressed as the percentage of eggs fertilized by each suspension. + = positive tests; 0 = negative tests.

Age of Sperm Suspensions	*A*				*B*				*C*			
	Sperm Extracts				Sperm Extracts				Sperm Suspensions			
	Egg-agglutination Tests				Fertilizin-inactivation Tests				Percentage of Eggs Fertilized			
					(200)	(100)	(50)	(25)				
	8%	4%	2%	1%	8%	4%	2%	1%	8%	4%	2%	1%
hours												
24	+	+	+	0	+	+	+	+	100	100	100	100
30	+	+	+	+	+	+	+	+	100	99	100	89
36	+	+	+	0	0	+	+	+	100	100	100	94
49	+	+	0	0	+	+	0	0	100	93	12	11
56	+	+	0	0	+	+	0	0	100	13	0	3
72	0	0	0	0	0	0	0	0	26	0	0	0

The data are recorded in Table V. Column *A* lists the egg-agglutination tests, column *B* the fertilizin-inactivation tests, and column *C* the fertilizing power of each suspension. Each horizontal row thus records comparable tests at various times. For the first thirty-six hours the active principles are present, in general, in all extracts except for the 1 per cent which has lost its capacity to agglutinate eggs. Throughout this period the sperm suspensions fertilize 89–100 per cent of the eggs. After forty-nine hours, both egg-agglutination and fertilizin-inactivation tests are negative in the 2 per cent and 1 per cent extracts and at this time the fertilizing power of the 2 per cent and 1 per cent sperm suspensions has dropped to 12 per cent and 11 per cent respectively. The active principles have disappeared in all extracts after seventy-two hours and the fertilizing power of each suspension has dropped to zero except for the 8 per cent suspension which fertilizes

26 per cent of the eggs. At the close of the experiment living sperm were present in each suspension (except the 1 per cent) as shown by clear cytoplasm of the sperm heads and slight motility.

Many sources of error are present in an experiment of this sort which cannot be adequately controlled. The necessity for testing the same solutions for three days introduces unavoidable contamination and the condition of the eggs and sperm used for testing varies widely in different urchins from day to day (Goldforb, 1929). Nevertheless, it is clear that the loss of fertilizing power of aging sperm suspensions parallels the loss of active principles from extracts of these suspensions.

The Effect of Sperm Extracts on Fertilization and Development

Effect of Sperm Extracts on Both Gametes

In an extensive series of experiments, normal and jellyless eggs were fertilized in sperm extracts (.1 per cent to 8 per cent), transferred to sea water after exposure to the extracts for from thirty seconds to three hours, and at various times counts were made of the percentage of eggs developing. Controls consisted of the same eggs fertilized in heated sea water. Fertilization and development in extracts below about 2 per cent concentration equals that of the controls but above this concentration marked inhibition occurs. The majority of ova remain unfertilized and no fertilization membranes appear despite the presence of numerous active sperm at the cortex. Those eggs which are activated usually cleave normally but cease developing at or before the non-motile blastula stage if allowed to remain in the extracts. When the embryos are transferred to sea water they develop into normal plutei. These effects occur in both jellyless and normal eggs but the inhibition is greater in the latter, probably due to agglutinated jelly which physically prevents escape of the blastulae from the egg-masses.

In these experiments both germ-cells were simultaneously exposed to sperm extracts and the block to fertilization could be due to the action of extracts on the ovum alone, spermatozoön alone or on both gametes. We will now consider the action of extracts on each gamete.

Effect of Sperm Extracts on the Fertilizing Power of Sperm

Sperm suspensions were made in sperm extracts and fresh eggs fertilized with these mixtures: 1 per cent sperm suspensions were prepared in .5 per cent to 8 per cent extracts and after exposure of sperm to the extracts for various times, one drop from each suspension was used to fertilize three drops of fresh eggs in 20 cc. sea water. The per-

centage of fertilized eggs was counted two hours after insemination. A 1 per cent sperm suspension in boiled sea water served as control.

TABLE VI

The inhibiting effect of sperm extracts on the fertilizing power of sperm. One per cent sperm suspensions prepared in sperm extracts of various concentrations. Figures give percentage of fresh eggs fertilized by one drop from each suspension after exposure of sperm to the sperm extracts for times given at left. Control = 1 per cent sperm suspension in boiled sea water.

Concentration of Sperm Extracts	8%	4%	1%	0.5%	Control
Time Sperm Exposed to Sperm Extracts	Percentage Eggs Fertilized				
5 seconds.....	18	1	68	—	
15 seconds.....	3	2	20	92	
45 seconds.....	8	3	17	100	
60 seconds.....	7	5	18	92	
15 minutes.....	3	3	32	12	
30 minutes.....	3	1	5	4	99
60 minutes.....	1	1	1	1	99

From Table VI, which gives the result of one experiment, it is seen that the percentage of eggs fertilized by sperm suspensions in extracts of above 1 per cent drops greatly after fifteen seconds at which time the 4 per cent suspension fertilizes only 2 per cent of the ova. After fifteen minutes exposure, the fertilizing power of the .5 per cent suspension has dropped from 92 per cent to 12 per cent; after thirty minutes the percentage of eggs fertilized by each suspension has fallen almost to zero. The controls fertilized 99 per cent of the ova after one hour. Numerous motile sperm were seen at the surface of the ova but fertilization membranes did not appear. Sperm extracts, then, block fertilization by a rapid inhibiting effect on sperm which lose their fertilizing power but retain motility. The loss of fertilizing power is directly proportional to both the concentration of extract and the time of exposure.

Effect of Sperm Extracts on the Fertilizability of Eggs

These experiments were run on jellyless eggs in order to permit direct contact between cortex and sperm extract and to eliminate any mechanical interference with fertilization due to agglutination of the egg jelly. Twenty drops of jellyless eggs from a single female were placed in 10 cc. sperm extracts (.1 per cent to 8 per cent) and at intervals ten

drops of eggs were transferred from each extract to 10 cc. sea water. The ova were fertilized immediately after transfer by two drops of a 1 per cent sperm suspension (made up fresh at the start of the experiment and used throughout) and the percentage of eggs developing was counted two hours later. Twenty drops of the same eggs in boiled sea water were treated in the same manner and served as control.

TABLE VII

The inhibiting effect of sperm extracts on the fertilizability of eggs. Jellyless eggs added to sperm extracts of various concentrations, transferred to fresh sea water, and immediately inseminated with fresh sperm. Figures give percentage eggs fertilized after exposure to the extracts for the times given at left. Control = same eggs in boiled sea water.

Concentration of Sperm Extracts	8%	2%	0.5%	0.1%	Control
Time Jellyless Eggs Exposed to Sperm Extracts	Percentage Eggs Fertilized				
minutes					
15	20	57	91	91	
30	24	45	90	98	
60	15	17	89	96	100
90	21	21	87	97	
120	2	8	66	93	92

The data of a typical experiment are given in Table VII. After two hours exposure to extracts of above 2 per cent, less than 10 per cent of the eggs are fertilized. At this time less inhibition occurs in the eggs exposed to the .5 per cent extract since 66 per cent are fertilized. The .1 per cent extract equals the controls (91–98 per cent fertilized). We may conclude that the inhibiting effect of sperm extracts on the fertilizability of eggs is due to an effect on the cortex and is directly related to the concentration of extract and length of exposure.[2]

Effect of Sperm Extracts on Fertilized Eggs and Embryos

We have seen that fertilization and development of eggs fertilized in sperm extracts are inhibited when the gametes are exposed to extracts

[2] Glaser (1914) stated that eggs which have been treated for two hours with "boiled sperm infusion" remain unfertilized when heavily inseminated. He believed this inhibition to be due to "purple X" since boiled sperm infusions from which the color had been removed did not block fertilization. Woodward (1915) recorded similar results. In the present study it was found that the presence of coloring matter did not appear to influence in any way the inhibiting action of sperm extracts on the fertilizability of eggs.

during their union in fertilization. The inhibiting effect of sperm extracts also occurs *before* the union of the germ-cells, involving egg and sperm individually. We will now consider the action of sperm extracts on eggs *after* normal fertilization.

Jellyless and normal eggs were inseminated in sea water, transferred to sperm extracts (8 per cent to .1 per cent) ten minutes after fertilization, and some of the eggs were transferred back to sea water after exposure to the extracts for from thirty seconds to one hour. Controls were the same eggs in sea water. The following observations were made:

Three hours after fertilization—Normal cleavage in all extracts equal to that of controls.

Seven hours after fertilization—Development of eggs in extracts of above 2 per cent concentration stopped at non-motile blastula stage. Healthy motile blastulae found in remaining extracts and controls.

Twenty-three hours after fertilization—Embryos in 8 per cent extract have cytolysed at the blastula stage. Rare plutei in the 2 per cent extract with majority cytolyzed at blastula stage. Healthy plutei found in remaining extracts and controls.

Throughout the series the ova transferred to sea water developed into normal plutei, indicating that the inhibitory effect of sperm extracts is reversible. In the concentrated extracts normal eggs were inhibited to a slightly greater degree than jellyless eggs due to agglutinated egg-masses preventing escape of the blastulae.

When a drop of sperm extract is added to a drop of free-swimming plutei in Chinese ink, the embryos are paralysed at once on entering the extract and fall to the bottom. Peristaltic movements of the gut and feeble ciliary motion are the only indications that the embryos are alive. Under high power the cilia are stuck together by their tips and the plutei are surrounded by clumps of ink particles enmeshed in the agglutinated cilia. Due to the increased stickiness of the cilia, the plutei adhere together in large clusters when a glass needle is moved through the solution. This phenomenon occurs in plutei which have developed from completely jellyless eggs and so is not the result of agglutination of particles of egg jelly by the sperm extract.

In attempting to explain these findings it must be realized that extracts of whole sperm undoubtedly contain numerous unknown substances but the action of the egg-agglutinating substance accounts for many of the above facts. We have seen that eggs fertilized in sperm extracts or placed in extracts after fertilization in sea water proceed

normally to the non-motile blastula stage and then abruptly stop developing. It is likely that the cilia, which are pushed forth from the surface of the blastula at this time, are immediately agglutinated by the extract with the result that the blastulae are unable to break out of the fertilization membranes and die *in situ*. When embryos are transferred to sea water from the extract, the cilia are able to function, motility follows, and development continues normally. Paralysis of motile embryos by sperm extracts is probably also caused by agglutination of the cilia.

Parthenogenesis with Sperm Extracts

Repeated attempts were made to initiate development in unfertilized *Arbacia* eggs by exposing them to sperm extracts. Both normal and jellyless eggs were immersed in extracts of various concentrations (.5 per cent to 100 per cent) for from five seconds to twenty-four hours and then transferred to sea water. In other experiments eggs were treated with mixtures of egg-water and sperm extracts, extracts from which the active principles had been removed by boiling or aging, dialysed extracts, etc. The results were entirely negative in all instances. It is clear that immersion of eggs in sperm extracts is not sufficient stimulus for development. Perhaps local application of sperm extracts may be effective.

Discussion

We have seen that there are two major effects of sperm extracts, one upon the surface of the egg and the other a reaction with fertilizin, which paralleled each other under various experimental conditions. It is not possible to determine whether these effects represent the activity of two distinct substances or two properties of a single substance since the indicators for each are different; eggs for the egg-agglutinating substance and fertilizin for the fertilizin-inactivator. It will be assumed that a single " active substance " is present in sperm extracts which reacts with eggs and fertilizin.

The question of chief importance relating to this study is whether the active substance functions in fertilization. This substance is extractable only from sperm, agglutinates *Arbacia* eggs and embryos but has no effect on *Arbacia* sperm or blood corpuscles, and combines specifically with fertilizin which is believed to play an important part in fertilization. This marked degree of tissue specificity between eggs, fertilizin, and the active substance suggests that the latter is related to fertilization.

Because sperm loses its fertilizing power before loss of motility, Lillie (1915*b*) postulated that sperm contains a " fertilizing substance "

which combines with fertilizin and thereby activates the ovum. Such a substance, if present in sperm extracts, must theoretically (1) combine with fertilizin and neutralize its capacity to agglutinate sperm, (2) be lost with the fertilizing power of sperm, and (3) activate unfertilized eggs. Since sperm extracts inactivate fertilizin in quantitative proportions and loss of fertilizing power of sperm suspensions parallels loss of the active substance from extracts of these suspensions, two of the above criteria are fulfilled. On the other hand, sperm extracts will not activate eggs, which would tend to disprove the similarity of the fertilizing and active substances. It is possible that the active substance is non-diffusible and hence cannot permeate the cortical membrane, local application is necessary to cause activation, or a penetrating force (sperm) is essential to carry the fertilizing substance into the cytoplasm. Fertilization might then result from injecting sperm extracts into the cortex or increasing the permeability of the vitelline membrane. The block to fertilization caused by sperm extracts is additional evidence against the identity of the active and fertilizing substances. Assuming that fertilizin is necessary for activation, the inhibiting effect of sperm extracts on the fertilizability of eggs may be due to the neutralization of fertilizin at the egg surface by the active substance of sperm extracts. Possibly agglutination of the peripheral layer of the cortex physically prevents sperm penetration.

Lillie (1919, p. 219) suggests that there is a high degree of chemical specificity in the union of egg and sperm and Just (1930, p. 331), defending Lillie's use of immunological terms in his fertilizin theory, states, ". . . The biology of fertilization has more points in common with immune reactions than with any other biological phenomenon." The active substance of sperm extracts resembles bacterial agglutinins in certain respects. The following properties of bacterial agglutinins are taken from Wells (1929) and Kolle and Hetsch (1935): (1) Agglutinins disintegrate in sera when preserved, are non-dialysable, pass only incompletely through Chamberland filters, and are not destroyed by extracting serum with lipoid solvents. (2) Electrolytes are necessary for agglutination reactions, which will not occur in distilled water. The cation and not the anion of the added salt is of importance. The reactions take place over a wide range of hydrogen-ion concentration (pH 3.7–9.0). (3) The specificity of agglutination reactions is less marked than that of other immune reactions due to the complex structure of cells which permits similar proteins, carbohydrates and lipids to occur in cells of different species; different species of bacteria will thus react with a single agglutinin. (4) Dead bacteria agglutinate as readily as living ones and the agglutination of living bacteria does not kill them. In each

of the above properties there is a remarkable similarity between the agglutination of bacteria by sera and the agglutination of eggs by sperm extracts. Certain differences are also found; thus all known agglutinins are destroyed at or below 70° C. whereas the egg-agglutinating substance resists boiling for hours.

One of the theories concerning the mechanism of agglutination is that the agglutinin reduces the electronegative charge which in part keeps bacteria (or other cells) in suspension, thereby rendering them more susceptible to the precipitating action of salts. We have pointed out that egg-agglutination occurs only in the presence of certain cations (Mg or Ca) and according to Heilburnn (1923), the surface of *Arbacia* eggs is negatively charged. Possibly the active substance of sperm extracts reduces this negative charge at the surface of the ovum and permits the cations of the electrolyte to precipitate the eggs. The agglutination membrane would then form by precipitation of dissolved colloids at the periphery of the jelly, which has been "sensitized" to the precipitating action of salts by the active substance.

The active substance appears to be related to fertilizin in that both substances are non-dialyzable, thermostable, colloidal, and do not give the usual protein tests. It is likely that the active substance and fertilizin belong to a class of chemical compounds related to, if not identical with, bacterial agglutinins.

In conclusion, the remarkable tissue specificity of the active substance, its ability to combine selectively with fertilizin, its agglutinating action on eggs and embryos, its relation to the fertilizing power of sperm, and finally, the analogies between immune reactions, fertilization, and the properties of the active substance, all suggest that this substance plays a definite rôle in the fertilization reaction.

Summary

1. Sperm extracts of *Arbacia,* prepared by heating and filtering sperm suspensions in sea water, irreversibly agglutinate eggs of the same species. The reaction is characterized grossly by a dense coagulation of the ova and microscopically by the appearance of an "agglutination membrane" at the periphery of the egg jelly. Sperm extracts agglutinate both jelly and cortex of unfertilized, fertilized, living or dead ova.

2. The "egg-agglutinating substance" is fixed by the jelly and removed from solution. It is found only in extracts of sperm and cannot be extracted from any other cells or tissues of the sea-urchin, is not secreted by sperm into sea water, and is not present in extracts of old sperm. The egg-agglutinating substance is colorless, non-dialysable,

colloidal, highly thermostable, disintegrates on standing, is preserved best at low temperatures, and does not give the usual protein tests. On extracting sperm with hot or cold alcohol-ether, the egg-agglutinating substance is found in the protein residue but not in the lipid extract. Mg and Ca ions are the only ions of sea water which are essential to the extraction of the egg-agglutinating substance, no specific anions taking part in the process. Sperm extracts made in distilled or tap water are inert.

3. Sperm extracts inactivate the sperm-agglutinating power of *Arbacia* fertilizin, the capacity for inactivation varying directly with the concentration of extract. The "fertilizin inactivator" has many properties in common with the egg-agglutinating substance. Loss of fertilizing power from aging sperm suspensions parallels the loss of the egg-agglutinating substance and fertilizin inactivator from extracts of these suspensions.

4. Sperm extracts block fertilization in *Arbacia* by a direct effect on each gamete:

(*a*) Sperm suspensions in sperm extracts rapidly lose their fertilizing power as measured by their ability to fertilize fresh eggs in sea water. The extracts thus block fertilization by a direct action on the spermatozoön.

(*b*) The fertilizability of jellyless eggs is greatly decreased after exposure to sperm extracts, which exert an inhibitory effect on the cortex.

(5) *Arbacia* embryos are instantly paralysed and agglutinated by sperm extracts. Eggs which are fertilized in extracts cease developing before the motile blastula stage but this effect is partially removed by transferring the eggs to sea water.

6. All attempts to activate *Arbacia* eggs by immersion in sperm extracts have been unsuccessful.

7. It is suggested that the agglutination of eggs and the inactivation of fertilizin by sperm extracts represent two effects of a single active substance extracted from sperm. Theoretical and experimental evidence is presented that this substance probably plays a definite rôle in the fertilization reaction.

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